Overexpression of α-Tubulin Suppresses But Does Not Inhibit the Growth of the Fission Yeast *Schizosaccharomyces pombe*

Tetsuya Horio*, Akiko Taniwaki, Miyuki Shimizu and Chie Mori

Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan

Abstract: Microtubules are multifunctional and essential cellular organelles that are mainly composed of α- and β-tubulin dimer proteins. While it is very well known that the level of tubulin protein is strictly controlled in higher eukaryotes, tubulins can be overexpressed to toxic levels in certain fungal species. In this manuscript, we report that α-tubulin can be overexpressed by using a strong inducible promoter in the fission yeast *Schizosaccharomyces pombe*. α-Tubulin was overexpressed to 22-fold more than wild-type level. The growth of the strain overexpressing α-tubulin was significantly slower than a wild-type control. Immunofluorescent staining showed eventual accumulation of α-tubulin near the nuclei.

INTRODUCTION

It is very well known that expression levels of tubulin, the main building block of the microtubule, are strictly regulated by post-translational regulation machinery in animal cells [1,2]. The tubulin level regulation system in fungal organisms has also been studied, however, observations of different species led to different conclusions. In the filamentous fungus *Aspergillus nidulans*, overexpression of α- and β-tubulin genes was attempted by placing these genes downstream of a strong promoter [3]. Attempts to overexpress either one of tubulin genes resulted in only a marginal increase of the protein products although the levels of messenger RNA of the tubulin genes increased dramatically after induction of the overexpression. Co-overexpression of α- and β-tubulin genes resulted in significant (2-4-fold), but rather small compared to the level of messengers (50-100-fold), increase of protein products [3]. These observations strongly suggested the presence of some kind of regulation system to maintain tubulin levels in *A. nidulans*. In this fungus, excess tubulin monomers, either α- or β-, are removed before they become toxic to growth, or transcription is inactivated before producing toxic levels of monomers. In the budding yeast *Saccharomyces cerevisiae*, tubulin genes could be overexpressed by hooking the tubulin genes downstream of a strong promoter [4]. This observation indicated that, unlike the case of animal cells or *A. nidulans*, each tubulin could be overproduced and could cause a negative effect on the growth of *S. cerevisiae*. In both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, overexpression of β-tubulin is toxic for cell growth [4-8]. While overexpression of β-tubulin exhibited similar, but not identical, toxicity in these yeasts, overexpression of γ-tubulin, the third member of tubulin superfamily, had a different effect on the growth of these yeasts. In *S. pombe*, overexpression of γ-tubulin using a strong promoter resulted in 160-fold overexpression of the protein and cell lethality [9]. In *S. cerevisiae*, however, up to 300-fold overexpression of Tub4 protein, the γ-tubulin equivalent protein, did not affect the growth [10]. Increased gene dosage of the α-tubulin gene in *S. pombe* did not affect the growth [11,12]. This fact suggested the presence of a regulation system to prevent overproduction of the α-tubulin.

To further understand the effect of α-tubulin overexpression in *S. pombe*, we attempted forced-overexpression of α-tubulin by placing its gene under the control of a strong inducible promoter. Overexpression of α-tubulin in *S. pombe* resulted in a decrease of proliferation rate but not total inhibition of the growth. The overproduced α-tubulin eventually accumulated to the peripheral region of nuclei after a prolonged period of overexpression.

EXPERIMENTAL PROCEDURES

Strains and Media

S. pombe haploid strains HM123 (*h*$^-$*, leu1-32*), MB93 (*h*$^-$*, leu1*-32*, ura4*-D18) and MB94 (*h*$^+$*, leu1*-32*, ura4*-D18) were used. YE (1% yeast extract, 3% dextrose, and 1.6% agar for solid medium) was used as complete medium. EMM2 [13] was used as minimal medium. 50 μg/ml of leucine, 50 μg/ml of uracil and 10 μg/ml of thiamine were added to the medium when needed. For media containing the anti-microtubule agent thiabendazole (TBZ), TBZ was added from a 20 mg/ml stock solution in dimethylsulfoxide. Cell number in the culture was counted using a hemocyto counter (Nihon Koden, Tokyo).

Construction of Plasmids and Strains

The *S. pombe* α1-tubulin gene (*nda2*$^+$) was amplified from the pNDA2.1 plasmid [14] by PCR using a set of primers # 64 (5'- GCATATGAGAGAAGTAATTTCTG-3') and # 110 (5'- GCTCGAGTTAATACTCTTCGTCAGC-3') so as to add a NdeI restriction site and a XhoI site at each end of the coding sequence respectively. Amplified DNA was purified, digested with NdeI and XhoI and, then, ligated to

*Address correspondence to this author at the Institute of Health Biosciences, the University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan; Tel: +81-88-633-9357; Fax: +81-88-633-9105; E-mail: horio@nutr.med.tokushima-u.ac.jp and kaerugen@mac.com

1874-4370/08

the downstream of the inducible *nmt1* promoter in the vector pREP1 [15,16] and was named pMS635. The vector pREP81, which is essentially the same vector as pREP1 except for weakened *nmt1* promoter activity [16] was used as a no-induction control vector.

Induction of Overexpression

For comparison of growth profile, each HM123 transformant of pMS635, pNDA2.1 and control vector pREP81 was first cultured in EMM2 liquid medium supplemented with 10 μg/ml of thiamine, thus repressing conditions for *nmt1* promoter, until exponential phase. Cell density of each culture was measured using a hemocyto counter. Cells were harvested by centrifugation and washed twice with EMM2 liquid medium and, then, suspended in EMM2 without thiamine, thus inducing conditions for *nmt1* promoter, and cultured at 32°C with gentle shaking. The growth was monitored by sampling a small fraction of each culture and counting the cell density using the hemocyto counter.

Quantification of Overproduced α-Tubulin

Production of α-tubulin was monitored by western blotting using an anti-α-tubulin monoclonal antibody TAT1 [17]. The strain carrying pMS635 was cultured under the inducing conditions, cells were harvested in a time course manner and crude extracts were prepared by crushing cells using glass beads. Cell density of each sample was adjusted to 5 x 10^5 cells/μl. The western blotting was performed by following the standard procedure and signals were detected by chemiluminescence using an ECL Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Signals were recorded using a Luminescent Image Analyzer LAS-1000 (Fuji Photo Film, Tokyo, Japan). For quantitative western blotting, a serial dilution method [9] was used. In brief, series of dilutions of crude extracts from the strain carrying pMS635 and pREP81 were prepared, the α-tubulin was detected by western blotting, and each band was quantified to compare the expression levels.

Quantification of Transcripts by Real Time-PCR

Cultures of the strains carrying pMS635 under inducing conditions, pNDA2.1 and pREP81 were harvested and total RNA of each strain was isolated by disrupting the cells using glass beads in the presence of phenol: chloroform (1:1 ratio). Harvested cells were suspended in RNA extraction buffer (50 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, pH 8.0) and an equal volume of phenol:chloroform was added. The cell suspension was mixed with the glass beads and cells were crushed by vortexing vigorously. The aqueous phase was recovered, extracted by phenol: chloroform once again and, then, precipitated by adding ammonium acetate and ethanol. Isolated RNA was stored at -30°C until use. To synthesize the first strand DNA corresponding to the population of mRNA in the cell, reverse transcription was carried out by using the isolated RNA, PrimeScript RTase (Takara, Japan) and the oligo-dT primer supplied by the manufacturer. Real-time PCR amplification was performed by using SYBR Premix Ex Taq (Takara, Japan) and Applied Biosystems 7500 (Applied Biosystems Japan). The primers used for amplification were as follows; *S. pombe* α-tubulin-specific primers (# 205; 5'- CAGGCTGCCGTTACTAGC-3', # 55; 5'- ACTACCAGGAACGTGTTG-3'), primers corresponding to the *S. pombe* actin gene (*act1*$^+$) sequence (# 204; 5'-CGGTATTCATGAGGCTAC-3', # 161; 5'-CAATACCGG GATACATAG-3'). The sizes of DNA fragments expected to be amplified by these primer sets were 114 bp and 113 bp, respectively. Relative expression levels of α-tubulin were standardized by the level of actin mRNA.

Disruption of α2-Tubulin Gene

The second α-tubulin gene of *S. pombe,* the α2-tubulin gene (*atb2*$^+$) was disrupted by one-step gene replacement [18]. A genomic DNA fragment containing the *atb2*$^+$ gene was amplified by using primers designed to correspond to the 5' and 3' flanking sequences of *atb2*$^+$ gene (# 98; 5'-CTTTTTGCAAGCAATG-3'; # 99; 5'- CTTTACAAACA-GAAAG-3') and ligated into a TA-cloning vector pGEMT-Easy (Promega). One of resulting plasmids was digested with HindIII, which occurred in the coding sequence of *atb2*$^+$ gene, and the *S. pombe ura4*$^+$ marker gene was inserted. The resulting plasmid (pMS541) was linearized by digesting with SphI and PstI, transformed into MB93, and Ura$^+$ transformants were selected. Disruption of *atb2* gene in the transformants was confirmed by the supersensitivity to TBZ [11].

Immunofluorescence Microscopy

HM123 transformants carrying pMS635 was incubated in EMM lacking thiamine, thus inducing α-tubulin overexpression, aliquots of culture were sampled in a time course fashion, and fixed and processed for immunofluorescent staining. Immunofluorescent staining was carried out by following the procedure described previously [19]. The anti α-tubulin monoclonal antibody TAT1 [17] was used for microtubule staining. Cy-3 conjugated goat anti mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were preadsorbed with an *S. pombe* acetone powder [20] and used as secondary antibodies. Antibody stained cells were stained with 0.2 μg/ml 4'6-diamidino-2-phenylindole dihydrochloride hydrate (DAPI) in PBS and mounted on a coverslip.

RESULTS

Over the course of evolution, living organisms have developed numerous ways to regulate the level of cellular proteins. The tubulins are one of the most typical instances of protein level regulation in many organisms. Previous observations suggested that, while overexpression of β- or γ-tubulins was toxic in fission yeast *S. pombe*, overdosage of the α-tubulin genes has much less, if any, effect on the growth of *S. pombe*. However, whether or not α-tubulin exhibited any toxicity when expressed at very high levels, had not been previously tested.

Overexpression of α-Tubulin by Using a Strong Inducible Promoter

To determine if α-tubulin could be overexpressed in *S. pombe*, we attempted transient overexpression of the α-tubulin gene. We constructed a plasmid in which the *S. pombe nda2*$^+$ gene, the major α-tubulin gene [14], was placed downstream of the strong and inducible *nmt1* promoter [16]. We transformed the resulting plasmid, pMS635, into an *S. pombe* strain, HM123. Transformants of pMS635 grew as well as control transformants on EMM2 plates sup-

plemented with thiamine (repressive conditions for *nda2*$^+$ overexpression.) However, in the absence of thiamine, the growth of pMS635 transformants was significantly slower than control transformants (Fig. **1A**) indicating that overexpression of the α-tubulin in *S. pombe* negatively affects growth. To examine the degree of growth suppression caused by the α-tubulin overexpression, we set up EMM2 liquid cultures of one of pMS635 transformants and transformants of control plasmids; one of the strains carried the vector pREP81 and the other carried a plasmid containing *nda2*$^+$ gene sequence with its own promoter (pNDA2.1). Each strain was cultured till mid-exponential phase in EMM2 supplemented with thiamine and then transferred to EMM2 lacking thiamine (inducing conditions). It is known that peak level expression of an *nmt1* promoter-controlled gene is achieved 12-15 h after the depletion of thiamine from culture media [16]. The growth rate of the pMS635 carrying strain was dramatically reduced 15-16 h after the start of induction (Fig. **1B**). The growth rate of this strain was comparable to the growth rate of one of control strains carrying the vector (mean doubling time; 3 h 15 min) up until 15-17 h after the start of induction. This result indicated that suppression of the growth took place when or slightly after the induced expression level got to the peak. Although the growth rate of the pMS635 carrying strain was greatly reduced, the strain continued to grow at a slow but relatively steady rate (mean doubling time; 13 h 30 min). This is quite different from the cases of β- or γ-tubulin overexpression. In both of these cases, complete growth inhibition was observed (for example, see Fig. (**2**) of ref 9). On the other hand, the strain carrying pNDA2.1 grew constantly at the rate slightly slower than the other control strain (mean doubling time; 4 h 15 min). This result indicated that carrying extra copies of the α-tubulin gene causes a slight growth defect which is not distinguishable from the sizes of colonies formed on a solid medium (Fig. **1A**).

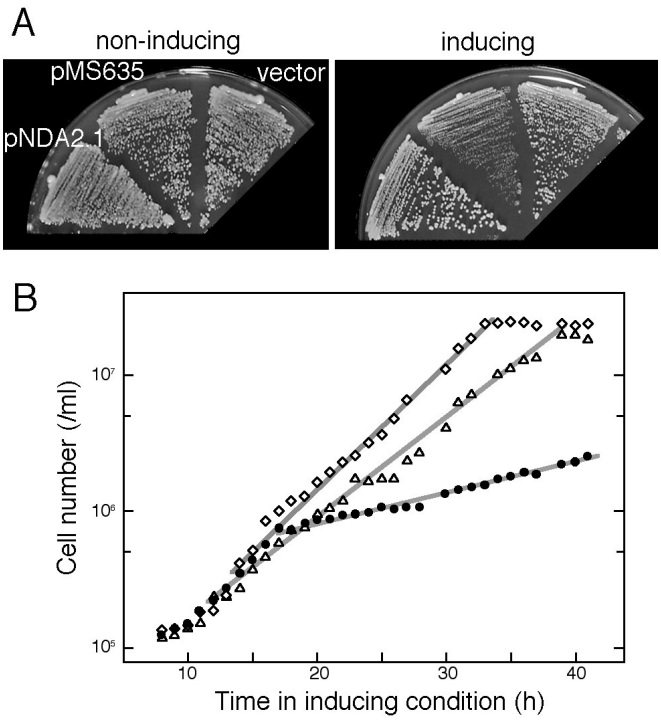


Fig. (1). Growth of α-tubulin overexpressing strains. (**A**) Strains carrying a plasmid containing the α1-tubulin gene with its own promoter (pNDA2.1), under the control of *nmt1* promoter (pMS635), and the vector were streaked on non-inducing medium (left) and the medium that induces the *nmt1* promoter activity (right) and incubated at 32°C for 3 days. (**B**) Growth profile of the strains carrying the vector (open rectangles), pNDA2.1 (open triangles) and pMS635 (filled circles) in liquid inducing medium at 32°C.

α-Tubulin is 22-Fold Overexpressed in a pMS635 Carrying Strain

It is known that most eukaryotes have some kind of system to maintain the level of the α- (and β-) tubulin. To investigate if this is the case for *S. pombe,* we examined the level of α-tubulin in the pMS635 carrying strain by western blotting using an anti-α-tubulin monoclonal antibody, TAT1. A significant level of overexpression of α-tubulin was observed in the time course cell extract samples (Fig. **2A**).

Overexpression of α-tubulin was observed in the sample of 15 h after the start of induction and in those taken at later time points. This result indicated that, by using the strong inducible *nmt1* promoter, α-tubulin can be overexpressed to a significant level in *S. pombe* and the growth defect observed in the overexpressing strain coincided with the elevated level of the α-tubulin. To quantify the level of overexpression, we prepared serial dilutions of crude extracts of the pMS635 carrying strain incubated 21 h in inducing conditions and the strain carrying the vector. Each dilution was blotted, probed with TAT1 and the signal was quantified (Fig. **2B**). By plotting the intensity of each band, we found that the pMS635 carrying cells contain about 22-fold more α-tubulin molecules per cell than wild-type. On the other hand, the strain carrying pNDA2.1 did not exhibit an increase of the α-tubulin amount detectable by western blotting (data not shown).

mRNA Level is 5-Fold Higher than Protein Level

Overproduction of the α-tubulin protein could be explained by one of the following two ways. One possibility is that the α-tubulin was overexpressed following the increase of mRNA which was produced under the control of the strong *nmt1* promoter, thus, there is no control system for the α-tubulin level in *S. pombe*. The other possibility is that the amount of mRNA was too much to maintain the level of protein product within the normal range for *S. pombe* tubulin-level regulation system in the pMS635 carrying strain. If the latter is the case, more α-tubulin mRNA than α-tubulin protein should be present in this strain. To test these possibilities, we performed real-time PCR to quantify the level of mRNA in each strain. We isolated total RNA from each of the pMS635, pNDA2.1 and pREP81 carrying strains, synthesized cDNA by reverse transcription and performed real-time PCR amplification. The relative amount of α-tubulin mRNA in each strain was standardized by the amount of actin mRNA. As shown in Fig. (**3**), the strain carrying pMS635 contained 120.0 ± 4.1 times more α-tubulin mRNA than the control strain, while the strain carrying pNDA2.1 had 9.5 ± 0.3-fold more. In both pMS635 carrying and pNDA2.1 carrying strains, the levels of the α-tubulin mRNA were signifi-

cantly higher than the elevated levels of their final protein products.

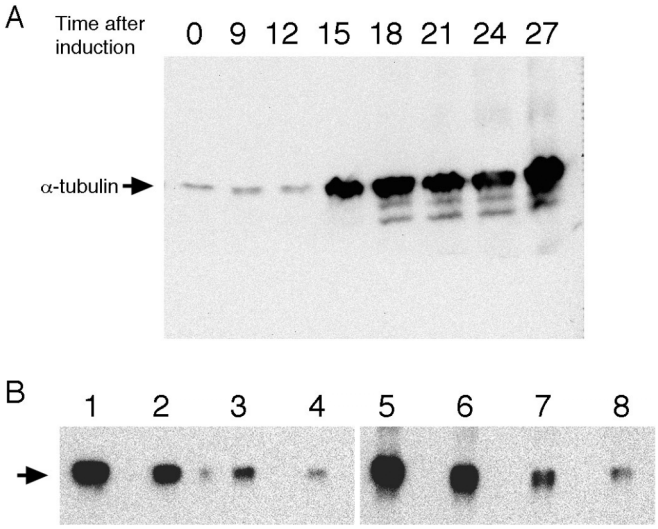


Fig. (2). Detection and quantification of overexpressed α-tubulin by western blotting. (**A**) Time course samples of the strain carrying pMS635 were prepared at the time points after the start of induction indicated above of each lane. The concentrations of crude extracts were adjusted to equal cell number (5 x 10^6) and loaded onto each lane. Blotted samples were probed with the monoclonal anti-α-tubulin antibody TAT1. Crude extract prepared from the cells in the uninduced condition was loaded onto the left-most lane and indicated as time point 0. (**B**) Serial dilutions of crude extracts prepared from the strain carrying the vector (lanes 1-4) and the strain carrying pMS635 incubated in inducing conditions for 21 h (lanes 5-8) were loaded and probed with TAT1. The crude extracts were diluted by half each time. Lane 1 represents the signal from the crude extract equivalent of 2.5 x 10^6 cells while lane 5 contains 1.5 x 10^5 cells-equivalent amount of crude extract.

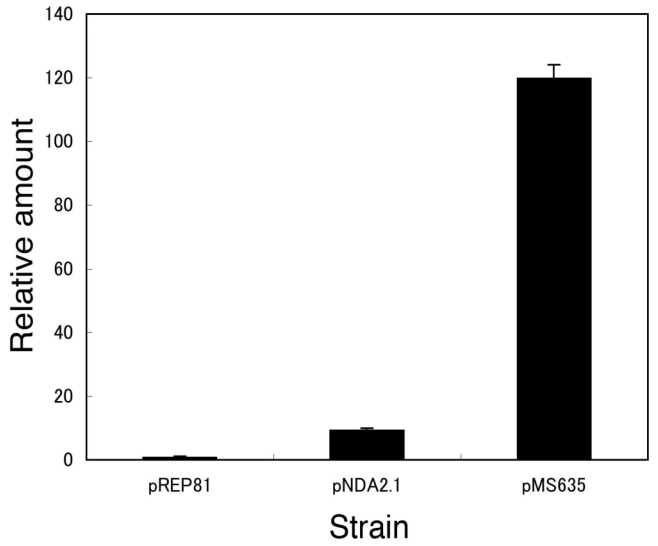


Fig. (3). Comparison of mRNA levels by real-time PCR. Relative amount of α-tubulin mRNA of each strain was plotted on the graph. cDNAs prepared from total RNAs of the strain carrying the vector (pREP81), pMS635 or pNDA2.1 were used and the amount of α-tubulin mRNA in each strain was standardized to the amount of actin mRNA. Bars indicate standard deviations from the average.

Cytological Phenotypes

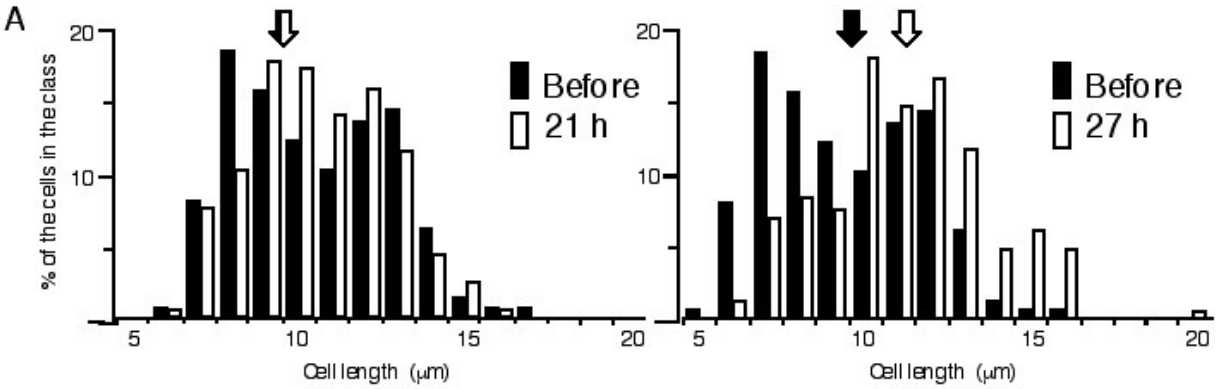
We explored the mechanism of the growth defect caused by the overexpression of α-tubulin by observing cytological phenotypes of the α-tubulin overexpressing strain. Since fission yeast grows only lengthwise, the length of the cells reflects the cell cycle stage of that particular cell [13]. We measured the cell length distributions of the pMS635 carrying strain under the inducing conditions. Although the growth of the strain was significantly suppressed by 15 h after the start of induction, the average cell length did not change significantly till 21 h after the start of induction (9.5 ± 2.1 μm) compared to the average cell length before the induction (9.4 ± 2.3 μm). The average cell length started to increase after that time point (27 h; 11.2 ± 2.5 μm). The distribution of cell lengths changed according to the time incubated in the inducing conditions. At 21 h after the start of induction, although the average cell length did not change significantly, a decrease in the shorter cell population was observed (Fig. **4A**, left panel). At 27 h after the start of induction, this tendency was more apparent and accumulation of longer cells was observed (Fig. **4A**, right panel). These results suggested the accumulation of the cells in a later stage of the cell cycle, in late G2 or in M phase.

Chromosomes of *S. pombe* occupy a hemispherical area of nucleoplasm throughout interphase and condensation of chromosomes is observed only transiently during the mitotic phase. In the cells overexpressing α-tubulin, both a decrease of normally dividing nuclei and the appearance of abnormal chromosome morphology, which indicates a defect in the mitotic phase, were observed while the fraction of cells with interphase chromosome morphology stayed relatively unchanged over the course of observation (Fig. **4B**). This observation, along with the change in cell length distribution, suggested that, in the cells overexpressing the α-tubulin, the mitotic process is delayed and somewhat defective which results in accumulation of larger cells and appearance of aberrant chromosome morphology.

We further examined the defective phenotype by observing the localization of α-tubulin in the cells by anti-α-tubulin staining using TAT1. Fifteen hours after the start of induction, which corresponded to the initiation of growth suppression, no particular staining structure was observed in the vast majority of the cells (81% of total). Normal cytoplasmic arrays of microtubules and mitotic spindle microtubules were observed only in 7% of the cells counted. Accumulation of TAT1 stained bodies was observed in small fraction of the cells and the number of cells with this phenotype increased as incubation time went on. patch abutted to the nucleus (Fig. **5G**,**H**, indicated by arrows). Despite the extensive defect in microtubule organization, we did not observe significant morphological alterations such as bent or branched cells in the culture.

Effect of Anti Microtubule Reagent on α-Tubulin Overexpressing Strains

Many anti-microtubule agents bind to the α-/β-tubulin dimer, essentially decrease the active pool of tubulin dimer and, as a consequence, destroy the microtubule. To examine how the overexpression of the α-tubulin affects drug sensitivity, we tested the growth of the strains carrying pREP635



B

Chromosome mitotic indecies

	normal				abnormal			
	interphase	mitotic	post mitotic	septated	stretched	compact	condensed chr	n
before	83.5	6.4	5.9	4.2	0	0	0	407
12 h	74.9	12.1	5.5	7.4	0	0	0	363
15 h	86.9	7.3	4.7	6.7	0	0	0	358
18 h	89.5	2.3	1.9	2.1	4.2	0	0	427
21 h	83.8	3.2	0.4	4.3	6.8	1.5	0	470
24 h	81.4	1.8	0	3.8	7.0	3.8	2.3	398
27 h	80.3	3.7	0	2.4	4.7	5.9	3.0	507

Fig. (4). Cell length distribution and Chromosome morphology of α-tubulin overexpressing strain. (**A**) Cell length distribution of the strain carrying pMS635 in inducing conditions for 21 h (left) and 27 h (right). Cell length distributions are presented by open bars along with the cell length distribution of the cells in the culture before induction (filled bars). The averages of the cell lengths of this strain before the induction (filled arrows), 21 h and 27 h after the start of induction (open arrows) are indicated at the top of each graph. (**B**) Chromosome morphology of the strain carrying pMS635 in inducing conditions. Chromosome morphology of DAPI stained cells were counted and categorized into normal (left of broken line) and abnormal (right).

or the vector on EMM2 (inducing medium) containing various amounts of the anti-microtubule agent TBZ.

To assess the effect of the drug, we used a strain MS1 in which one of the α-tubulin gene (*atb*2^{+}) was disrupted. It has previously been shown that the *atb*2 disruptant is fully viable but becomes supersensitive to TBZ supposedly because of the shortage of the tubulin molecule [11]. The transformants of pMS635 with wild-type background (the strain we have been using) and *atb*2 disruptant background were tested for TBZ sensitivity. While we failed to detect a significant effect of TBZ on the growth of the strains of wild-type background, partial suppression of TBZ supersensitivity was observed in MS1 transformants (Fig. **6**).

DISCUSSIONS

Tubulin Level Control in Fungal Cells

The microtubule is essential for all eukaryotic cells and the cellular concentration of the tubulin dimer, its main building block, must be maintained at appropriate level to assure the dynamic properties of the microtubules. In mammalian cells, extensive studies have shown that a strict post-transcriptional control system regulates the level of available tubulin subunits in the cytoplasmic pool [1,2,21]. On the other hand, several preceding studies have indicated that the tolerance for overexpression of tubulin genes in fungal cells varies from one organism to another. In the filamentous fungus *Aspergillus nidulans*, overexpression of α- and β-tubulin was attempted by placing these genes under the control of a strong promoter [3]. When either the α-or the β-tubulin alone was induced for overexpression, no apparent increase of protein product was observed. When, however, both the α- and the β-tubulin were induced for overexpression in the same cell, 2-4-fold increase of both the α- and the β-tubulin level was observed. In both cases, tubulin mRNA levels were elevated 50-100-fold more than normal levels in inducing conditions. These results indicated the presence of strong posttranscriptional regulation of tubulin level in *A. nidulans*.

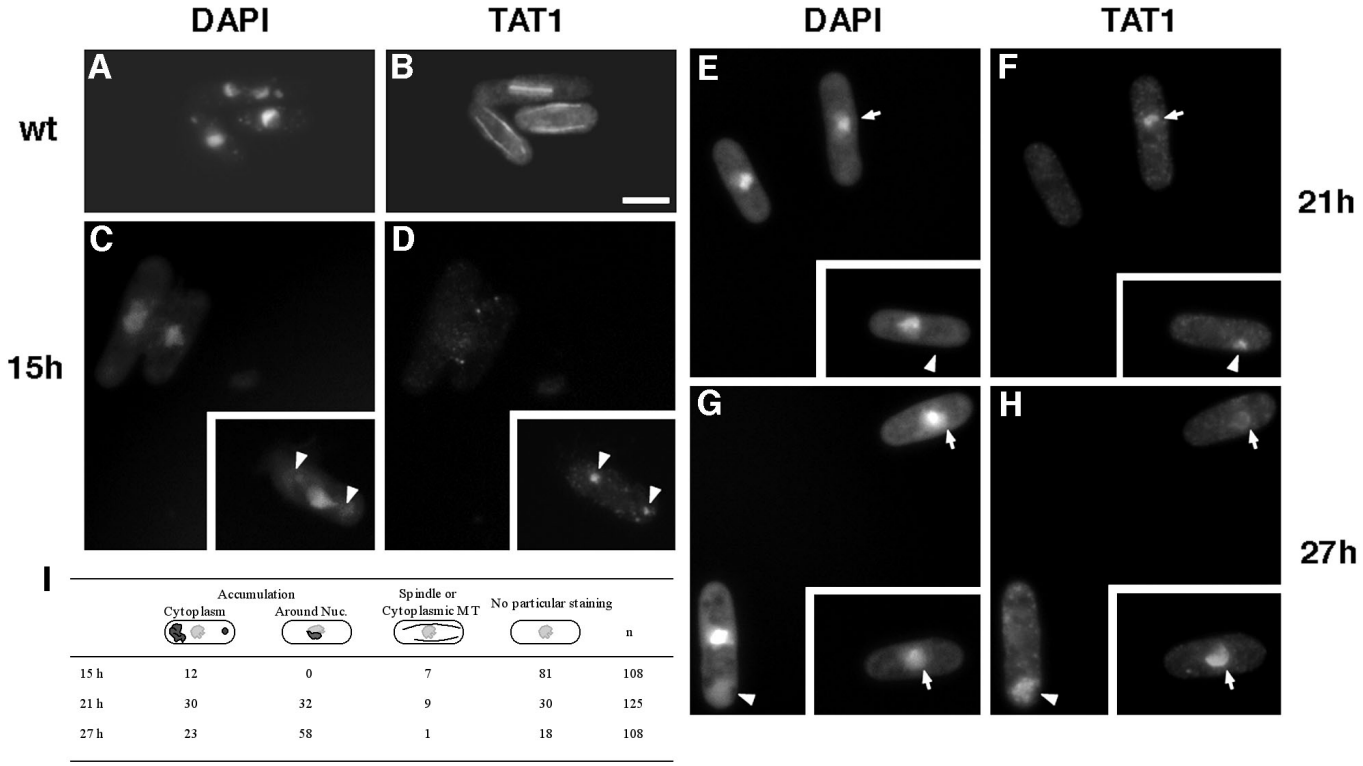


Fig. (5). α-Tubulin staining patterns of overexpressing strain. Staining patterns of chromosomes and α-tubulin of wild-type (**A**,**B**) and the strain carrying pMS635 in inducing conditions for 15 h (**C**,**D**), 21 h (**E**,**F**) and 27 h (**G**,**H**) are shown. Chromosomes and α-tubulin were visualized by DAPI and the anti-α-tubulin antibody TAT1 respectively. Bar in B = 5 μm and is good for panels A-H. Accumulation of TAT1 staining bodies in the cytoplasm and near the nuclei are indicated by arrowheads and arrows respectively. (**I**) Time course change of the distribution of the α-tubulin-staining pattern was summarized.

In the budding yeast *S. cerevisiae*, it has been reported that overdosage of the β-tubulin gene is lethal [4-6,22]. Contradictory results have been reported on the effect of the α-tubulin overexpression. Burke *et al.* [22] reported that overexpression of α-tubulin under the control of a strong inducible promoter resulted in 20-fold overproduction of mRNA, less than 2-fold overexpression of protein product and no obvious effect on the growth of the strain. On the other hand, Weinstein and Solomon [4] reported that α-tubulin under the control of the same promoter could overproduce the protein up to 30-fold and cause reduction of growth rate and viability. It has also been indicated that, unlike the case of the β-tubulin, moderate overpresence of the gene or mRNA of the α-tubulin affected neither the level of the α-tubulin protein nor growth rate [5,22]. Thus, *S. cerevisiae* does have some system to control the level of tubulin protein within a viable range when gene dosage is moderately altered. In the fission yeast *S. pombe,* it has been reported that overdosage of the β-tubulin gene caused by introducing the β-tubulin gene in a high copy number plasmid was lethal [7,8]. On the other hand, it has been reported that α-tubulin cannot be overexpressed by increasing the gene dosage both at the mRNA level and at the protein level [11,14]. This observation indicated the presence of a regulatory system to maintain α-tubulin levels in *S. pombe* [12]. There are two α-tubulin genes in the *S. pombe* genome, *nda2*$^+$and *atb2*$^+$. These proteins are 86% identical. Both proteins are expressed during vegetative growth and are functionally interchangeable, i.e. a recessive mutant of *nda2*$^+$ can be suppressed by overproduction of *atb2*$^+$[12,14].

From our results of real-time PCR experiments, the strain carrying pNDA2.1, which carries the *nda2*$^+$ gene with its own promoter in a multi-copy vector, contains about 10-fold

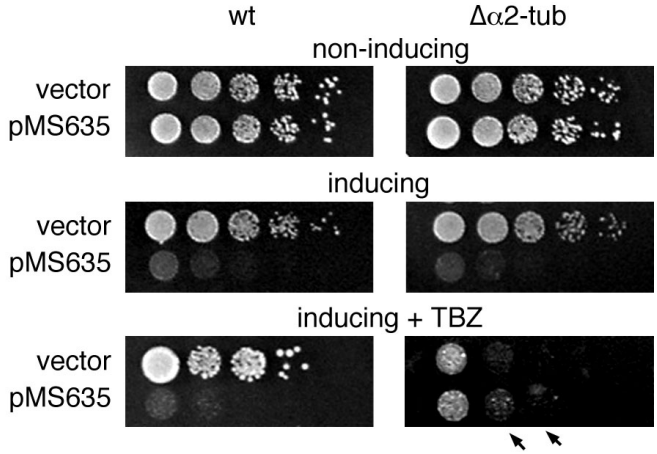


Fig. (6). Effect of anti-microtubule agent on different α-tubulin overexpressing strains. Strains carrying pMS635 or the vector with wild-type background (wt; left panels) or with α2-tubulin disruptant background (Δα2-tub) were spotted as 5-fold dilution series on the non-inducing (top panels) or inducing (bottom two panels) media and incubated at 32°C for 3 days. The anti-microtubule agent TBZ was added at 12.5 μg/ml to the medium in bottom panel.

more *nda2*$^+$ mRNA than the control strain. This small increase of mRNA may have been below the detection range of previous observations [11,14] which reported that the *nda2*$^+$ mRNA level was kept about the same regardless of the presence or absence of extra copies of the gene as judged by northern blotting. While the increase of mRNA by 10-fold did not affect the protein levels significantly, the 120-fold increase of mRNA resulted in 22-fold increase of final protein product. Overproduction of α-tubulin up to this level caused reduction of the growth rate. Our results are very much comparable to the results of overexpression of the α-tubulin in *S. cerevisiae* reported by Weinstein and Solomon [4]. The five-fold difference between mRNA level and protein level in both of these cases could indicate that the α-tubulin level is regulated by limiting the production of protein at a posttranslational stage in these yeasts. It is worth noting that *A. nidulans,* a filamentous fungus, is more tolerant of increased α-tubulin mRNA. A 100-fold increase of α-tubulin mRNA did not significantly affect the level of α-tubulin protein in this fungus [3]. *A. nidulans* may have a greater capacity to handle excess mRNA than yeasts or the machineries controlling the level of tubulins may differ among these fungi.

Growth Suppression Caused by α-Tubulin Overexpression and Accumulation of α-Tubulin Around the Nuclei

The strain overexpressing α-tubulin exhibited specific phenotypes. First, growth was strongly suppressed but never stopped during the observation period. This is a quite distinct response compared to the effects of overexpression of other tubulin genes. Moderate overexpression of β-tubulin was toxic in fission yeast [7,8] and 160-fold overexpression of γ-tubulin could inhibit proliferation completely and reduce viability dramatically [9]. This fact indicated that excess α-tubulin protein at this level is not extremely toxic for *S. pombe*. The accumulation of larger cells (Fig. **4**) and cells with microtubule defects (Fig. **5**) were observed in the culture of the α-tubulin overexpressing strain. These phenotypes indicate that the mitotic process is taking longer than in the wild-type. The strain carrying pNDA2.1, the *nda2*$^+$ gene plasmid with its own promoter, exhibited a slight delay of growth, although the α-tubulin protein level was not increased detectably in this strain. The delay of growth could be caused by a slight increase of the α-tubulin protein. If this is the case, *S. pombe* is quite sensitive to the increase of the α-tubulin level.

The accumulation of α-tubulin was observed. Since the fraction of cells accumulating α-tubulin increased well after the growth suppression, the accumulation of α-tubulin cannot be the cause of the decrease of growth rate. At this point, we cannot readily explain the mechanism for the accumulation of α-tubulin to particular locations of the cell and there could be several possibilities to explain this phenomenon. One possible explanation for the accumulation of α-tubulin to the nuclear region is by the overflow of the nuclear trafficking system. Since fission yeast cells undergo nuclear division with the presence of an intact nuclear envelope throughout its process, tubulin molecules must be transported into the nucleoplasm to form mitotic spindles. Upon the completion of nuclear division, tubulin molecules are exported from the nucleoplasm to the cytoplasm to accommodate the reorganization of the interphase cytoplasmic microtubule array. The presence of excess α-tubulin could unbalance this import/export system resulting in the eventual accumulation of α-tubulin in nucleoplasm.

ACKNOWLEDGEMENTS

We would like to thank Dr. Keith Gull for providing the TAT1 antibody, Dr. Mitsuhiro Yanagida for strains and plasmids and Dr. Kinsey Maundrell for pREP plasmids. We also would like to thank Liz Oakley for critically reading the manuscript. This work had been supported in part by a grant from the Sumitomo Foundation.

REFERENCES

[1] Cleveland DW. Autoregulated instability of tubulin mRNAs: a novel eukaryotic regulatory mechanism. Trends Biochem Sci 1988; 13: 339-43.

[2] Cleveland DW. Autoregulated control of tubulin synthesis in animal cells. Curr Opin Cell Biol 1989; 1: 10-4.

[3] Yoon Y, Oakley BR. Purification and characterization of assembly-competent tubulin from *Aspergillus nidulans*. Biochemistry 1995; 34: 6373-81.

[4] Weinstein B, Solomon F. Phenotypic consequences of tubulin overexpression in *Saccharomyces cerevisiae*: Difference between alpha-tubulin and beta-tubulin. Mol Cell Biol 1990; 10: 5295-304.

[5] Katz W, Weinstein B, Solomon F. Regulation of tubulin levels and microtubule assembly in *Saccharomyces cerevisiae*: consequences of altered tubulin gene copy number. Mol. Cell. Biol 1990; 10: 5286-94.

[6] Liu H, Krizek J, Bretscher A. Construction of a GALI-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. Genetics 1992; 132: 665-73.

[7] Hiraoka Y, Toda T, Yanagida M. The NDA3 gene of fission yeast encodes β-tubulin: a cold-sensitive nda3 mutant reversibly blocks spindle formation and chromosome movement during mitosis. Cell 1984; 39: 349-58.

[8] Javerzat JP, Cranston G, Allshire RC. Fission yeast genes which disrupt mitotic chromosome segregation when overexpressed. Nucleic Acids Res 1996; 24: 4676-83.

[9] Horio T, Basaki A, Takeoka A, Yamato M. Lethal level overexpression of γ-tubulin in fission yeast causes mitotic arrest. Cell Motil. Cytoskel 1999; 44: 284-95.

[10] Marschall LG, Jeng RL, Mullholland J, Stearns T. Analysis of Tub4p, a yeast tubulin-like protein: implication for microtubule-organizing center function. J. Cell Biol 1996; 134: 443-54.

[11] Adachi Y, Toda T, Niwa O, Yanagida M. Differential expressions of essential and nonessential α-tubulin genes in *Schizosaccharomyces pombe*. Mol Cell Biol 1986; 6: 2168-78.

[12] Yanagida M. Yeast tubulin genes. Microbiol Sci 1987; 4: 115-18.

[13] Mitchison JM. Physiological and cytological methods for *Schizosaccharomyces pombe*. Methods Cell Physiol 1970; 4: 131-65.

[14] Toda T, Adachi Y, Yanagida M. Identification of the pleiotropic cell division cycle gene NDA2 as one of two different α-tubulin genes in *Schizosaccharomyces pombe*. Cell 1984; 37: 233-42.

[15] Maundrell K, Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene 1993; 123: 127-30.

[16] Basi G, Schmidt E, Maundrell K. TATA box mutations in the *Schizosaccharomyces pombe nmt1* promoter affect transcription efficiency but not the transcription start point or thiamine repressibility. Gene 1993; 123: 131-6.

[17] Woods A, Sherwin T, Sasse R, MacRae TH, Baines AJ, Gull K. Definition of individual components within the cytoskeleton of *Trypanosoma burucei* by a library of monoclonal antibodies. J. Cell Sci 1989; 93: 491-500.

[18] Rothstein AH, Lade BN, Chui D, Lin S, Dunn JJ, Studier FW. Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 1983; 56: 125-35.
[19] Horio T, Oakley BR. Human γ-tubulin functions in fission yeast. J. Cell Biol 1994; 126: 1465-73.
[20] Harlow E, Lane D. Antibodies: A laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1988.
[21] Gonzalez-Garay ML, Cabal F. α-Tubulin limits its own synthesis: Evidence for a mechanism involving translational repression. J. Cell Biol 1996; 135: 1525-34.
[22] Burke D, Gasdaska P, Hartwell L. Dominant effect of tubulin overexpression. Mol. Cell. Biol 1989; 9: 1049-59.

Received: December 14, 2007 Revised: March 12, 2008 Accepted: March 12, 2008